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# A Celebration of the New Head and an Evaluation of the New Mouth

Minireview

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**Twenty years ago now, Carl Gans and Glen Northcutt proposed that the main invention of vertebrates was a new head, with its full array of sensory organs involved in an active predatory lifestyle. Tracing back the embryological origin of these structures, they showed how all are primarily derived from the neural crest and the placodes, two transient ectodermal cell populations in the embryo. These cell types were then used for further innovations, such as a new mouth in jawed vertebrates. The interplay between patterning and plasticity of the neural crest is largely responsible for the endless variation of vertebrate craniofacial features in evolution.**

"…from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

**—Charles Darwin, *The Origin of Species*, 1859**

The origin of vertebrates has long attracted the attention of naturalists and zoologists and has experienced a much deserved renewal of interest in recent years. In this regard, it has benefited enormously from the emergence of evolutionary developmental biology (evo-devo) as a new discipline, one that has established its own conceptual framework and experimental tools and has brought together expertise and discoveries from traditionally distant fields, such as paleontology, developmental biology, and genomics. The underlying concept behind this approach is that the evolutionary changes in morphology must have been caused by changes in the genetic programs responsible for the development of these morphological traits.

### *Of Heads*

When, 20 years ago, Carl Gans and Glen Northcutt came up with the stunning and provocative new head theory (Gans and Northcutt, 1983), evolutionary developmental biology was just being born as a new discipline. However, this theory was not fully incorporated into the discussion of the developmental mechanisms of evolution until a decade later. This had a lot to do with the reemergence of the cephalochordate amphioxus as a model for comparative molecular embryology, it being the closest invertebrate relative of the vertebrates (Holland, 2000). The appearance of the neural crest and the placodes provided the basis for the formation of paired sense organs and structures at the anterior part of the head, which represented a new vertebrate unit and allowed an active predatory lifestyle. These new cellular types gave rise to new structures that are characteristic of the vertebrate head. But what have we really learned about the evolutionary origin of the neural crest and placodes in vertebrates over these 20 years? We have accumulated a fair amount of information about the genes involved, but only recently have we started to get some glimpse of the underlying mechanisms. Unfortunately, our understanding has not progressed equally for both cell types, as our knowledge of placode evolution is still lagging behind what we know about the neural crest.

The neural crest cells appear in the most dorsal aspect of the neural tube from where they detach via an epithelial-to-mesenchymal transition and then migrate to the periphery. Once they have reached their destinations, the neural crest cells differentiate into various cell types. In the cranial region, it contributes to the cranial ganglia and is responsible for the formation of the craniofacial skeleton (Le Douarin and Kalcheim, 1999). The specification of the crest is linked to the specification of the dorsal character of the neural tube. Studies of gene expression patterns in amphioxus and ascidians have revealed that the genetic system involved in establishing the dorso-ventral polarity of the neural tube is strictly conserved across chordates. This involves ventral expression of genes like *Shh* or *HNF3*-β and dorsal expression of members of the *Pax*, *BMP*, and *Snail* family (see Figure 1). Therefore, even if nonvertebrate chordates do not have neural crest, much of the genetic regulatory systems required for neural crest formation were in place before vertebrates appeared (Holland and Holland, 2001). The dorsal expression of *Snail* in amphioxus and *Ciona* is of particular interest, as the product of this gene could represent the link between neural crest specification and the acquisition of migratory behavior in vertebrates. Cells that express *Snail* family members in the vertebrate neural tube lose their contacts with neighboring epithelial cells and acquire the capability to migrate as individual cells. Recent work has shown how *Snail* is involved in the regulation of this particular cellular phenotype, triggering what has been called the epithelial-to-mesenchymal transition (Nieto, 2002). However, in ascidian and amphioxus, *Snail*-expressing cells never leave the neural tube. It would be interesting to know whether this is because some of the downstream targets of *Snail* have been recruited anew in the vertebrate lineage or because even if amphioxus *Snail*-expressing cells could migrate, the environment does not provide the necessary clues to trigger this process. Learning more about how the epithelial-to-mesenchymal transition of the crest cells occurs in vertebrates will allow us to decipher at which step *Snail*-expressing cells are trapped in the prechordate neural tube.

Another important issue is the possibility for variation that the neural crest offers in the vertebrate head. It has been long thought that the basic patterning information for craniofacial development was passively transferred

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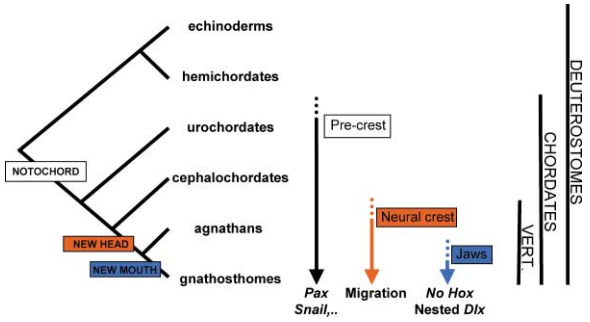


Figure 1. Diagram of the Evolutionary History of Deuterostomes and the Successive Steps Leading to Present Day Vertebrate Body Plan

Urochordates include ascidians such as *Ciona*, and cephalochordates refer to the lancelet amphioxus. The notochord is a defining feature (synapomorphy) of chordates, while the new head and neural crest is a vertebrate character, and the new mouth and elaborate jaw are specific of gnathostomes. Additions of gene networks or processes leading to morphological innovations are also depicted.

from the neural axis to the neural crest at its point of production. Migratory cells would carry this information to the periphery and would develop and differentiate according to their positional memory. Classic transposition experiments in chick embryos, also published 20 years ago, showed how neural crest cells would differentiate according to their original axial level and not to the new environment they would meet (Noden, 1983). When neural crest fated to form jaw elements was transposed to a more posterior location, it would result in ectopic jaw skeletal elements. This view has been challenged by the demonstration of an extremely high degree of plasticity in the final destiny and genetic program of neural crest cells and the importance of the migratory path that they follow (Trainor and Krumlauf, 2000). Even Noden's original grafting experiments have been reinterpreted as carrying a region of the neural tube with intrinsic signaling capability, the isthmus, that would be responsible for the results obtained (Trainor et al., 2002). The isthmus would inhibit key genes responsible for suppressing jaw formation (see below) in its new location, therefore allowing the appearance of the ectopic elements. This would mean that neural crest cells do not possess inherent patterning information but instead obtain it from the surrounding environment.

Further work has shown how fine tinkering of neural crest populations may be behind some of the classical examples of evolutionary theory, such as the morphological variation in birds' beaks. Since "The Origin of Species," variations in the size and morphology of the beak of the Galapagos finches have been a classroom example of evolution at work. It could well be the case that environmental impact on beak morphology (Grant and Grant, 2002) could be canalized through an inherent variability in key developmental aspects of the neural crest. Molecular studies have shown how the different elements of the beak are patterned by interplay between information carried by the neural crest and local signaling centers in the periphery (Lee et al., 2001). An inherent informative capacity of neural crest populations has been dramatically shown in recent experiments performed on quail and duck embryos that have very obvious differences in their beaks. Duck neural crest cells from the beak-forming region grafted into quail embryos result in duck-like beaks appearing on quails, and vice versa. Grafted neural crest cells will not only develop according to their species of origin, but will instruct surrounding host-derived ectoderm to grow and deploy a temporal-spatial gene expression program according to the donor species (Schneider and Helms, 2003). These results seem at odds with the "plasticity" view exposed above and would drive us back to a "pre-patterning" model where neural crest retain a memory about what they have to do and can even organize other tissues around it. However, there are major differences between both sets of experiments that could explain the apparent disparity of the results and reconcile both views. While in the first case (Trainor et al., 2002) we are dealing with intraspecies grafts, in the second (Schneider and Helms, 2003) we are looking at interspecies combinations. In addition, the size of the grafts used in the duck-quail transplantations could explain the organizing capability of the neural crest through community effects, by which big groups of cells maintain cell identity when grafted heterotopically (Trainor and Krumlauf, 2000). In summary, a fine balance ought to exist between plasticity and informative capability of crest populations, which together with the influence exerted by ectodermal signals will pattern and elaborate craniofacial morphology.

Where are the future areas of research going to take us? Surely we will need to compare whole gene regulatory networks between different organisms, as the genes themselves and the approximate spatial distribution of their products are a shared character of chordates. Much more information regarding how signaling and regulatory clues are integrated on cis-control elements necessary for neural crest production is needed before we can perform worthy comparisons with ascidians or amphioxus. Cross-species transgenic analysis of cis-regulatory elements among different chordates is a starting point in this direction (Manzanares et al., 2000). Similar experiments among vertebrates would also help to decipher the mechanisms leading the evolution of the regulatory elements in gene duplicates (Locascio et al., 2002). Only then will we be able to pinpoint the changes that occurred in the regulation of these genes concomitant with the appearance of the new head. Welcome additions to this area of research are the draft sequence of the genome from the ascidia *Ciona intestinalis* (Dehal et al., 2002) and the promise of an amphioxus genome project (http://tbx.wustl.edu/~amphbase/White.pdf). Nonetheless, the new head theory has provided a much needed theoretical framework to direct current research on neural crest evolution and its role in vertebrate origins.

### *Of Jaws*

Cranial neural crest originating from the midbrain and the hindbrain migrate in well-defined streams filling the branchial arches. The branchial arches can be thought of as bilateral ectodermal bags located ventrally in the cranial region of the embryo and which will later fuse along the midline to form the majority of the face and pharynx. Once in the arches, neural crest cells differentiate into, among other derivatives, the cartilage and bone that will form the craniofacial skeleton of vertebrates.

In basal vertebrates, represented nowadays by the hagfish and lamprey (agnathans = no jaws), the arches form a cartilaginous ring, the true "arch," positioned externally relative to the mesoderm, forming the pharyngeal basket where muscles and tendons used in ventilation and feeding attach (Mallat, 1996). All other extant vertebrates (gnathostomes = jawed mouth) present a modified version of the branchial arch derivatives. First of all, the cartilaginous arches are located internal to the mesoderm, due to changes in the disposition of the neural crest cells within the arch (Kimmel et al., 2001). These authors also proposed that in lampreys the first element of the series (the first or mandibular arch) somehow depicts a "mixed style." It develops both external and internal cartilages (the external and internal cartilage bars) generated, respectively, by crest cells that remained in a lateral position and by crest cells that initiated the outside-in movement typical of gnathostomes. Nevertheless, lampreys do not have jaws, and Mallat (1996) has argued that the formation of the jaws meant a profound reorganization of the oral vertebrate cavity.

The jaws are formed from the first arch by differential growth and patterning relative to the other arches, with the upper and lower parts deriving from the proximal and distal parts, respectively. In Mallat's view, the mouth of agnathans is located anterior to the first branchial arch, where a pre-oral cartilage is in charge of opening and closing this cavity. However, with the appearance of the jaws, the "old mouth" was pushed anteriorly (still evident as a slit in front of the jaws of sharks), and a new cavity appeared at the level of the first arch derivatives (the jaws) constituting the new mouth. In his hypothesis, the function of this new mouth would be to increase the ventilation needed for a more active metabolism rather than grabbing preys, a function that would have appeared secondarily.

The importance of this invention is obvious, as jaws allow a completely new lifestyle to develop, where the eating of prey was a must, and it resulted in the extraordinary success of jawed vertebrates (there are no terrestrial agnathans; all are gnathostomes). How this happened during vertebrate evolution is largely unknown, but a series of recent molecular and embryological studies have started to give some hints as to how it could have occurred. In this regard, lamprey is in the limelight as a model system, as the obvious outgroup of jawed vertebrates. The early development of the neural crest and pharyngeal arches of lampreys is generally similar to that of gnathostomes. Furthermore, it has been shown that homologous genes are acting in these processes, providing optimal tools for comparative studies (Kuratani et al., 2001).

What makes jaws look so different from other arches, even though they all come from the same repeated series of homologous elements? It appears that *Hox* genes are partly responsible, as there is an incompatibility between *Hox* expression and jaw development (see Figure 1). The first arch is the only one that does not express any *Hox* gene. The most anteriorly expressed *Hox* gene in gnathostomes is *Hoxa2*, with an anterior limit between rhombomeres 1 and 2 in the hindbrain, but interestingly, it is not expressed in the neural crest that originates from r2 and migrates into the first arch. It is only expressed in more posterior neural crest migrating into the second arch. Consistent with this, ectopic *Hoxa2* expression in the first arch of frogs or chicks inhibits jaw development (Creuzet et al., 2002, and references therein). In the reciprocal experiment, *Hoxa2* mutant mice show duplications of first arch skeletal elements in the second arch (such as the tympanic ring) but not of the jaw. These results seem to contradict the overexpression experiments, but it might be explained because other *Hox* genes are still expressed in the second arch crest of *Hoxa2* mutants (Gendron-Maguire et al., 1993) that could be sufficient to specifically suppress jaw formation without preventing the formation of other elements. Indeed, quantitative effects of *Hoxa2* dosage have been described for the development of second arch skeletal elements (Ohnemus et al., 2001), and similar mechanisms could be operating in this case.

We find a nice evolutionary correlate of these experimental observations when we look at *Hox* genes in agnathans. It has been recently reported how a group 6 *Hox* gene from lamprey is expressed up to the first branchial arch (Cohn, 2002), suggesting that jaw expansion would be held at bay through this *Hox* expression domain. Group 6 *Hox* genes do not have an anterior limit of expression in the cranial region of gnathostomes but rather a posterior limit in the trunk region, and a lamprey group 5 *Hox* gene shows a more posterior limit of expression. This implies that *HoxL6* shows a break in colinearity, a fundamental property of *Hox* clusters in all metazoans. It is noteworthy that this is not a peculiarity of lampreys, as it is also observed in amphioxus (Cohn, 2002), that obviously has no jaws nor branchial arches.

Naturally, we must ask what mechanism is responsible for the generation of the *Hox*-free domain in the first arch region that is created. Signaling from the isthmus could be doing the job, as it has been shown that FGF8 coming from the midbrain-hindbrain boundary can inhibit *Hoxa2* expression in its rostral domain (Trainor et al., 2002). Turning back to lamprey, this would mean that the isthmus region would not posses this signaling capability or that *HoxL6* is regulated in a different way and does not respond to it. The genetic network at the midbrain-hindbrain boundary has not been explored in lamprey embryos, although it is noteworthy that a *Fgf8/17* gene is expressed in a narrow band at the right location (Shigetani et al., 2002).

The absence of *Hox* genes from the first arch is only a permissive state for jaw development, and other players must be responsible for intra-arch patterning. Good candidates are the homeobox-containing *Dlx* genes (see Figure 1). Mammals posses three pairs of tandemly arranged *Dlx* genes that are expressed in nested domains along the proximal-distal axis of the branchial arches. *Dlx1/2* are expressed throughout the arch, while *Dlx5/6* are confined to more distal territories, and *Dlx3/7* are only expressed in the tip of the arches (Panganiban and Rubenstein, 2002). Therefore, there is a case that a *Dlx* code exists for the diversification of proximal-distal elements along the branchial arches (Depew et al., 2002). Experimental evidence in support of this model comes from mice where pairs of *Dlx* genes have been mutated simultaneously. While *Dlx1/2* double mutants show proximal defects in the first and second arches (Panganiban and Rubenstein, 2002), *Dlx5/Dlx6* double mutants

show a striking distal phenotype of the first arch. These mice exhibit a complete transformation of the lower jaw into a mirror-image upper jaw, together with a symmetrical snout (Beverdam et al., 2002; Depew et al., 2002).

What the *Dlx5/6* double knockout mice are telling us is that, at least at certain stages of mouth development, the only difference between upper and lower jaw is the expression of *Dlx5* and *Dlx6* and that both parts of the jaw share a largely common genetic program. In this sense, we can consider the transformation observed as truly homeotic, as has been suggested (Depew et al., 2002). But then we may also ask whether this mutation results in an atavism, or in other words, whether mirror-jaws are a condition of the past (Beverdam et al., 2002). In this scenario, nested *Dlx* expression would not be an essential need for the appearance or invention of the jaw but only for its remodeling and asymmetry.

Lampreys show no obvious restricted expression of any *Dlx* genes along the proximal-distal axis of the branchial arches (Kuratani et al., 2001; Cohn, 2002, and references therein), but then this would not be the reason for it not having jaws. We cannot forget that proximal-distal defects in skeletal branchial elements are not specific for the first arch or jaws but also occur in the second arch for both *Dlx1/2* and *Dlx5/6* mutants (Depew et al., 2002). However, the interpretation of this latter transformation may be more complex. In relation to this, it is also worth noting that, at least in the chick, the initial proximal-distal patterning information for neural crest that migrates into the first arch and that therefore provides jaw identity comes from the foregut endoderm. However, this tissue is only able to pattern *Hox* negative neural crest but not *Hox* positive cells (Couly et al., 2002; Creuzet et al., 2002). In this respect, lamprey *HoxL6* is expressed in the neural crest that migrates into the first arch (Cohn, 2002), in theory making it unresponsive to the jaw-identity instructive endodermal signals.

It could well be that inhibiting *Hox* expression in the first arch territory permits the over-growth of cartilaginous elements that would then be the substrate for jaw modeling based on differential expression of *Dlx* genes across the branchial arches. Going a step further downstream we know that while *Hoxa2* represses key genes for endochondral ossification in the second arch (Kanzler et al., 1998), *Dlx5* activates chondrocyte differentiation during endochondral ossification, at least in the limb (Ferrari and Kosher, 2002). A fine balance between the opposing activities of these genes could then be the basis for differentiation of the jaw and other skeletal elements of the vertebrate face.

***"…so simple a beginning…"***

We have tried to convey the extraordinary history of the origins of vertebrates in light of recent molecular and comparative data. The framework provided by Gans' and Northcutt's new head theory has allowed us to view amphioxus as a "vertebrate in waiting" (Conway-Morris, 2000), where all major genetic players are ready to launch the invertebrate-to-vertebrate transition. The fine tuning of gene regulation in the neural crest resulted in the invention of the modern mouth equipped with new tools like the jaws or the beak. We can rejoice with Darwin and admire how from a migratory bunch of cells "endless forms most beautiful and most wonderful have…evolved" in the craniofacial region of vertebrate animals.

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